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New Family of Ulvan Lyases Identified in Three Isolates from the Alteromonadales Order*

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Ulvan is the main polysaccharide component of the Ulvales (green seaweed) cell wall. It is composed of disaccharide building blocks comprising 3-sulfated rhamnose linked to D-glucuronic acid (GlcUA), L-iduronic acid (IdoUA), or D-xylose (Xyl). The degradation of ulvan requires ulvan lyase, which catalyzes the endolytic cleavage of the glycoside bond between 3-sulfated rhamnose and uronic acid according to a β-elimination mechanism. The first characterized ulvan lyase was identified in *Nonlabens ulvanivorans*, an ulvanolytic bacterial isolate. In the current study, we have identified and biochemically characterized novel ulvan lyases from three Alteromonadales isolated bacteria. Two homologous ulvan lyases (long and short) were found in each of the bacterial genomes. The protein sequences have no homology to the previously reported ulvan lyases and therefore are the first representatives of a new family of polysaccharide lyases. The enzymes were heterologously expressed in *Escherichia coli* to determine their mode of action. The heterologous expressed enzymes were secreted into the milieu subsequent to their signal sequence cleavage. An endolytic mode of action was observed and studied using gel permeation chromatography and ^{1}H NMR. In contrast to *N. ulvanivorans* ulvan lyase, cleavage occurred specifically at the GlcUA residues. In light of the genomic context and modular structure of the ulvan lyase families identified to date, we propose that two ulvan degradation pathways evolved independently.

Ulvales are green algae that proliferate in eutrophicated coastal waters. Members of this family are commonly grown and collected for food or feed, although currently most of the biomass is put to limited use (1, 2). Among the polymers synthesized by these algae, ulvan is the most abundant polysaccharide in the architecture of the cell wall (2). This complex water-soluble anionic polysaccharide represents up to 29% of the algae dry weight (3). Although the constituent ratio might vary according to algae growth conditions, it is mainly composed of 3-sulfated rhamnose (R3S),[3] D-glucuronic acid (GlcUA), L-iduronic acid (IdoUA), and D-xylose (Xyl) (2). Ulvan building blocks appear frequently as repeating disaccharides comprising either GlcUA or IdoUA linked to R3S, termed ulvanobiuronic acid A or B, respectively (2). In addition, disaccharide moieties made of R3S linked to Xyl occur in lower amounts.

Ulvan's unique chemical and physicochemical properties make it an attractive candidate for several applications in the food/feed, agriculture, pharmaceutical, and biomaterials industries (2). In contrast to polysaccharides extracted from brown and red algae (*e.g.* alginate and agar, respectively), which are widely used in industry for their gelling and thickening properties, polysaccharides of green algae are less exploited. Expanding our understanding of ulvan structure and its enzymatic degradation would enable more extensive biomass utilization.

Thus far, only a few ulvan-degrading enzymes have been isolated from both marine and terrestrial microorganisms. Some of them, like the glucuronan lyases isolated from *Sinorhizobium meliloti* (4) and *Trichoderma* sp. GL2 (5), possess limited ulvanolytic activity. The first ulvan lyase activity was found in a marine bacterium by Lahaye *et al.* (1), who employed the newly discovered enzyme extract to degrade ulvan for structural analysis. More recently, several bacterial strains capable of metabolizing ulvan were isolated from the feces of a sea slug, *Aplysia punctate*, fed with *Ulva*. In this way, the Gram-negative *Nonlabens ulvanivorans* PLR was identified (6, 7) and its genome was sequenced (8). A novel ulvan lyase was purified from *N. ulvanivorans* batch culture, sequenced, and heterologously overexpressed in *Escherichia coli*, and the enzyme's ability to depolymerize ulvan biochemically characterized (9). *N. ulvanivorans* ulvan lyase was reported to cleave ulvan at the β(1→4) glyosidic bond between R3S and GlcUA or IdoUA via the β-elimination mechanism. The proton at the C5 position is abstracted, regardless of its configuration (*syn* for IdoUA or *anti* for GlcUA) with the hydroxyl group at C4 (Fig. 1). The β-eliminative cleavage results in the formation of a reducing end on one fragment and an unsaturated ring (Δ, 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid) on the non-reducing end of the second fragment (10). Because the protein sequence of *N. ulvanivorans* ulvan lyase had no characterized homolog in the databases, it was considered the first representative of a new family of polysaccharide lyases.

Sequence encoding an unsaturated β-glucuronyl hydrolase belonging to the glycoside hydrolase family GH105 (based on the Carbohydrate-Active Enzymes (CAZy) database) was found in the vicinity of the ulvan lyase in the *N. ulvanivorans* genome. This enzyme was shown to cleave specifically the

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[3] The abbreviations used are: R3S, 3-sulfated rhamnose; GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; Xyl, D-xylose; CM, conditioned medium; GH, glycoside hydrolase.

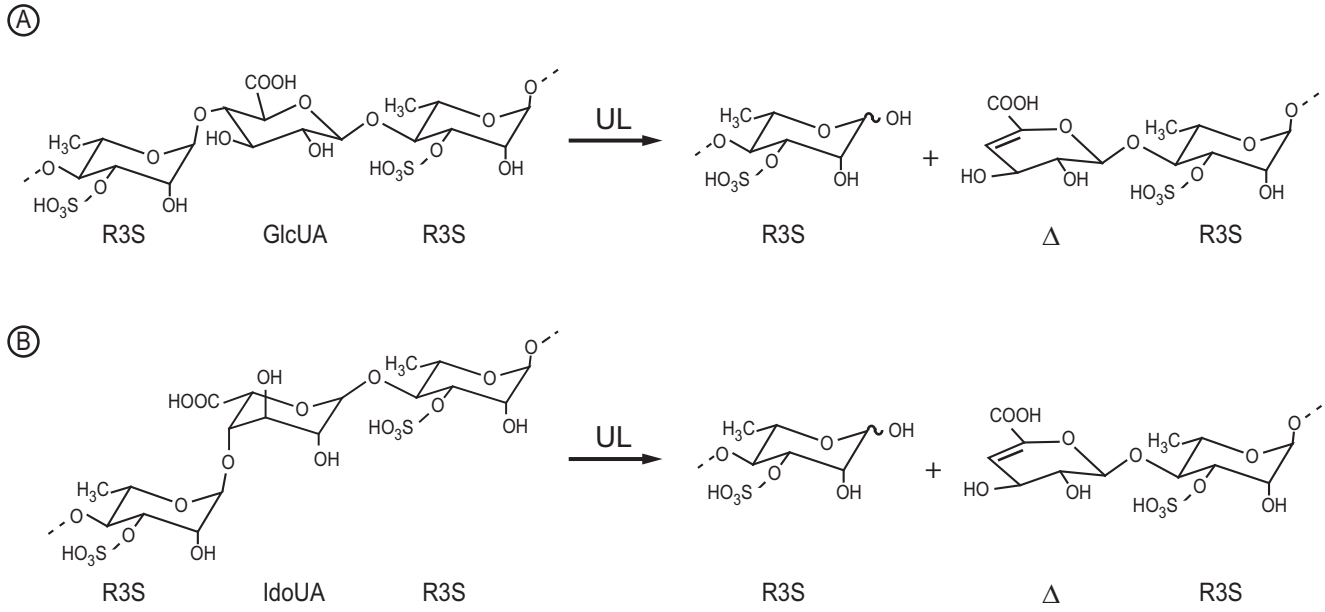

FIGURE 1. **Ulvan lyase mode of action.** *A* and *B*, chemical scheme of *N. ulvanivorans* ulvan lyase (*UL*) cleaving ulvan at the $\beta(1\rightarrow4)$ glyosidic bond between R3S and GlcUA (*A*) or IdoUA (*B*) via β-elimination mechanism. The β-eliminative cleavage results in the formation of a reducing end on one fragment and an unsaturated ring (Δ, 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid) on the non-reducing end of the second fragment.

unsaturated non-reducing end of the end products of the ulvan lyase (11). The spatial proximity within the genome of these two ulvan-degrading enzymes pointed to occurrence of polysaccharide utilization loci. With this premise in mind, we sequenced the genome of *N. ulvanivorans* and three additional ulvanolytic Alteromonadales isolates: *Alteromonas* sp. LOR, *Alteromonas* sp. LTR, and *Pseudoalteromonas* sp. PLSV (12, 13). Inspection of the genomes did not reveal genes encoding proteins homologous to *N. ulvanivorans* ulvan lyase. This led us to hypothesize that additional ulvan lyases that do not share sequence similarity with the known ulvan lyase may be encoded in the genomes of the three other ulvanolytic strains. Here, we report identification and biochemical characterization of four novel ulvan lyases, which belong to a new polysaccharide lyase family now being established[4] and are unrelated to *N. ulvanivorans* ulvan lyase.

Experimental Procedures

Strain and Plasmid Constructions—The bacterial strains and primers used in this study are listed in Tables 1 and 2, respectively. Native ulvan-degrading isolates were grown in Marine Broth (Difco) at 25 °C, whereas *E. coli* strains were grown in Luria Bertani (LB medium, Difco) at 37 °C. When appropriate, cultures were supplemented with antibiotics as indicated in Table 1. Primers were designed for amplifying genes of interest from the corresponding genomic DNA, with (*e.g.* LOR_107) and without their native signal peptide (*e.g.* LOR_107d) (Table 2). Both the insert and the expression vector were digested using the relevant restriction enzymes, gel-purified, and ligated to form a C terminus His-tagged protein. Recombinant plasmids were used to transform T7-expressing-competent *E. coli* (New England Biolabs) (Table 1).

Protein Expression and Purification—Transformed *E. coli* strains (Table 1) were used for the expression and purification of the His-tagged ulvan lyases. Batch culture was inoculated 1:50 (v/v) with transformed cells grown overnight and incubated for 2.5 h at 37 °C to reach optical density (600 nm) of 0.6 – 0.8. Protein expression was induced with 0.01 mM isopropyl 1-thio-β-D-galactopyranoside for 18 h at 16 °C. The induced culture was fractionated by centrifugation to obtain the culture supernatant containing secreted enzymes (herein termed conditioned medium, *i.e.* CM) and cell pellet as two separated fractions. After centrifugation, the pellet was resuspended in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5. Cells were lysed using a French press followed by centrifugation to remove bacterial debris. The resulting supernatant was applied to a nickel-Sepharose column charged with 100 mM $NiSO_4$ (GE Healthcare). After washing, the bound proteins were eluted with a linear gradient of imidazole ranging from 5 to 500 mM. The active fractions were pooled, and protein purification was verified using SDS-polyacrylamide gel electrophoresis stained with instant blue (Expedeon). Recombinant protein yields measured by NanoDrop 2000c (Thermo Scientific) were 2.03 mg/ml and 60 μg/ml purified from pET28a and pET22b constructs, respectively. Expression levels of His tagged proteins in CM and cell lysate fractions were analyzed by Western blotting using His·Tag monoclonal antibodies (Novagen) followed with ECL detection reagent (Thermo Fisher Scientific).

Enzyme Assay—Ulvan was provided by CEVA (Pleubian, France) and the polysaccharide was extracted from *Ulva rotundata* and was obtained as described by Nyvall Collén *et al.* (9). Screening for ulvan lyases by monitoring the formation of reducing ends was carried out using the ferricyanide method described by Lane and Lawen (14). Briefly, 2 g liter^{-1} ulvan in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5 was incubated in 1:1 (v/v) ratio with overexpressed cell lysate at 30 °C and sampled at

[4] B. Henrissat, personal communication.

TABLE 1
Strains used in this study

Strain	Relevant features	Reference
Ulvanolytic strains		
N. ulvanivorans PLR	Ulvan-degrading strain, genome as a template	6, 7
Alteromonas sp. LOR	Ulvan-degrading strain, genome as a template	12
Alteromonas sp. LTR	Ulvan-degrading strain, genome as a template	12
Pseudoalteromonas sp. PLSV	Ulvan-degrading strain, genome as a template	13
***E. coli* strains**		
T7 express competent *E. coli*/ pRIL (MK1466)	BL21 (DE3) enhanced derivate used for protein expression supplied with plasmid for rare codon usage, CmR	New England Biolabs/Stratagene
MK1466/pET22b	pET22b in MK1466, AmpR CmR	New England Biolabs/Novagen
MK1466/pET28a	pET28a in MK1466, KanR CmR	New England Biolabs/Novagen
MK1466/pMK2002	pET22b_LOR_61 in MK1466, AmpR CmR	This study
MK1466/pMK2018	pET22b_LOR_107 in MK1466, AmpR CmR	This study
MK1466/pMK2045	pET22b_PLSV_3875 in MK1466, AmpR CmR	This study
MK1466/pMK2046	pET22b_PLSV_3925 in MK1466, AmpR CmR	This study
MK1466/pMK1469	pET22b_PLR_UL in MK1466, AmpR CmR	This study
MK1466/pMK2040	pET22b_PLR_48 in MK1466, AmpR CmR	This study
MK1466/pMK2035	pET28a_LOR_107 in MK1466, KanR CmR	This study
MK1466/pMK2036	pET28a_LOR_107d in MK1466, KanR CmR	This study

TABLE 2
Primers used in this study
Restriction sites are underlined.

Number	Name	Sequence
MK1634	LOR_61_NcoI_F	GAGAGTCCATGGATATGAAATGTCTAAAAACATTGTTAGT
MK1635	LOR_61_BamHI_R	GAGAGGGATCCATTAGAACTACAGCGTGAATAAGTAC
MK1644	LOR_107_NcoI_F	GAGAGTCCATGGTCATGAAAATAAATCTAAGCATGCG
MK1645	LOR_107_XhoI_R	AGATCTCTCGAGCTCAGTAACACCCAAGTCG
MK1791	PSLV_3875_NcoI_F	AGAGAGCCATGGATATGAAATTAAATCTAAAAGCGAGTG
MK1792	PSLV_3875_XhoI_R	GAGAGTCTCGAGCCTAGAAACACCCAAATCAATAAC
MK1793	PSLV_3925_NcoI_F	AGAGAGCCATGGATATGAACGGTTTAAAAATGTTG
MK1794	PSLV_3925_XhoI_R	GAGAGTCTCGAGATTAGAGGTGCAGCGC
MK1156	PLR_UL_NcoI_F	GAGAGTCCATGGTGTTTTTTAAAGATTTATTCATCTTT
MK1157	PLR_UL_NotI_R	AGAGAGGCGGCCGCATTTACAATGAGCTTGGTTTGATA
MK1577	PLR_48_EcoRI_F	GAGAGTGAATTCAATGAGAAAATTAAAATATAATACTACTAGAG
MK1578	PLR_48_NotI_R	AGAGAGGCGGCCGCTTTACCTCATCTTTTGTATAGCTAG
MK1684	LOR_107d_NcoI_F	GAGAGTCCATGGCAAATGATAGCGTGTCG

various time points. Samples were mixed at 1:10 ratio (v/v) with ferricyanide solution composed of 300 mg liter^{-1} $K_3Fe(CN)_6$ (Sigma-Aldrich), 28 g liter^{-1} Na_2CO_3 (Sigma-Aldrich), and 1 ml liter^{-1} 5 M NaOH dissolved in purified water (Milli-Q). The mixture was boiled and cooled, and the amount of reducing ends was monitored by measuring absorbance at 415 nm. Characterization of the ulvan lyases was also performed by measuring the formation of double bonds in the assay at 235 nm as described previously (15). The spectrophotometer (Synergy™ 4 Hybrid Microplate Reader, BioTek) was set to read at intervals at the appropriate temperature. The assay solution was composed of 1 g liter^{-1} ulvan in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5 in 180 μl unless otherwise specified. 5 μl of purified enzyme (300 ng/ml) was added directly to the assay. The increase in absorbance at 235 nm was followed for up to 120 min.

Degradation kinetics of ulvan was measured by gel permeation chromatography after various incubation periods with 6 μg/ml purified LOR_107d. Ulvan solution was incubated with 60 μg/ml purified enzymes to obtain complete degradation into oligo-ulvan end products for ^{1}H NMR analysis. The assay solutions were composed of 100 g liter^{-1} ulvan in 0.1 M Tris-HCl, 0.2 M NaCl at pH 7.5.

Analytical and Semi-preparative Gel Permeation Chromatography—Ulvan degradation kinetics were monitored by analytical gel permeation chromatography. Samples were injected on a Superdex 200 (10/300 GL, GE Healthcare) and a peptide HR (10/300 GL, GE Healthcare) column coupled in series. Elution was conducted with an Ultimate 3000 HPLC system (Thermo Scientific, Dionex) operating at 0.5 ml min^{-1} with 50 mM $(NH_4)_2CO_3$ (pH 8) as the eluent. Detection of products was achieved with a Rapid Separation LC (RSLC) UV detector (Thermo Scientific, Dionex) operating at 235 nm, along with an IOTA 2 refractive index detector (Precision Instruments).

Oligo-ulvan end products were purified using filtered digested ulvan (0.22-μm nylon Clarinert syringe filters, Agela Technologies). Samples were injected on three Superdex 30 columns (GE Healthcare, 2.6 × 60 cm) mounted in a series. Elution was conducted at a flow rate of 1.5 ml min^{-1}. Oligosaccharides were collected with a Foxy R1 collector (Teledyne ISCO) controlled by AZUR software (Datalys).

NMR Spectroscopy—^{1}H NMR spectra were recorded at 298 K on a 400-MHz Avance DRX400 spectrometer (Bruker) in deuterated water as solvent and calibrated against the residual signal of the solvent. Prior to analysis, samples were exchanged twice in D_2O and re-dissolved in D_2O (99.97 atom % D). Chemical shifts are expressed in ppm in reference to an external standard (trimethylsilyl propionic acid). The HOD signal was not suppressed.

Results

Identification of Putative Ulvan Lyase Sequence in the Genome of Alteromonas sp. LOR—The genomes of the following four ulvan-degrading bacteria were sequenced and assem-

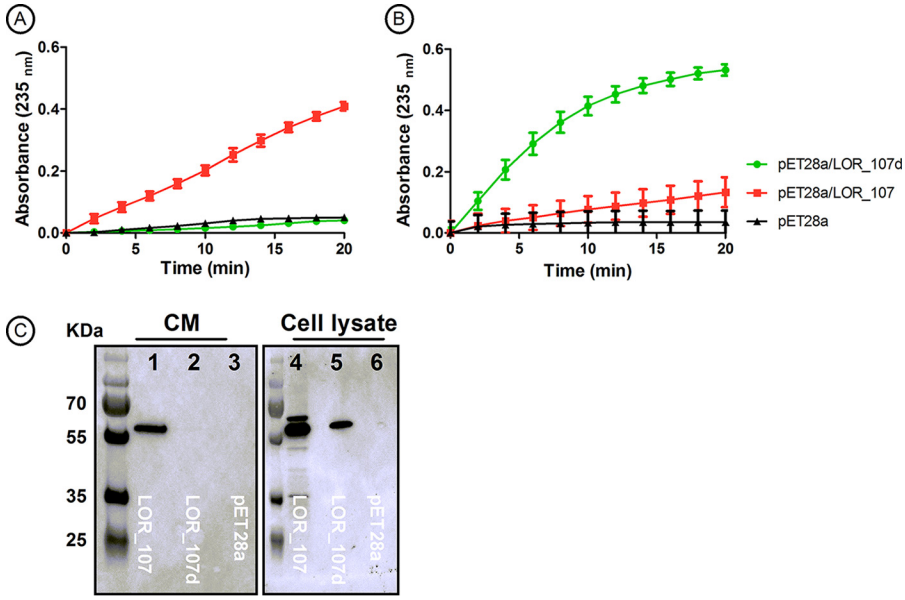

FIGURE 2. **Secretion of LOR_107 with and without signal peptide.** *A* and *B*, ulvan incubation with CM (*A*) or cell lysate (*B*) derived from overexpressed LOR_107 (*red*), LOR_107d (*green*, *i.e.* without signal peptide), and empty vector (*black*) was followed by UV (235 nm). Ulvan lyase activity is indicated by the increase in 235 nm absorbance. *Error bars* indicate ± S.E. *C*, Western blot of the CM fractions (LOR_107 (*lane 1*), LOR_107d (without signal peptide) (*lane 2*), and empty vector (*lane 3*)), and of cell lysate fractions (LOR_107 (*lane 4*), LOR_107d (*lane 5*), and empty vector (*lane 6*)).

bled: *N. ulvanivorans* PLR, *Alteromonas* sp. LOR, *Alteromonas* sp. LTR, and *Pseudoalteromonas* sp. PLSV, (8, 12, 13). Genome annotation was manually confirmed by CAZy[4] and revealed that these strains encoded many polysaccharide-degrading enzymes. For example, the genome of *Alteromonas* sp. LOR contained more than 60 glycoside hydrolases (GH) and polysaccharide lyases. Unexpectedly, genes coding for proteins with sequence homologous to the known ulvan lyase (9) were not detected. This suggested that degradation of ulvan in *Alteromonas* sp. LOR might be performed by a novel set of enzymes.

Inspection of the *Alteromonas* sp. LOR genome revealed that genes involved in the same metabolic pathway were grouped in clusters. Specifically, several GH- and sulfatase-encoding genes were co-located, making these clusters candidate sulfated polysaccharide utilization loci. Ten genes (WP_032096141.1, WP_032096210.1, WP_032096154.1, WP_032096198.1, WP_032096199.1, WP_052010186.1, WP_032096200.1, WP_032096220.1, KU168251, and WP_032096201.1) that code for hypothetical proteins located within these gene clusters or polysaccharide utilization loci were cloned, and the translated proteins were overexpressed using pET22b in *E. coli* cells (BL21). The soluble expressed proteins were incubated with ulvan, and degradation was monitored by detecting the formation of reducing ends (*i.e.* ferricyanide method (14)) and by measuring unsaturation at the non-reducing end (using UV at 235 nm). Strong degradation of ulvan was only observed for LOR_107 using both detection methods. Taken together, these observations suggested that LOR_107 is a lyase that cleaves the glycosidic bond to form an unsaturation, likely caused by the β-elimination reaction (data not shown).

LOR-107 Overexpression—Sequence analysis of LOR_107 indicated the presence of a 31-amino acid signal peptide, with a cleavage site between a threonine at position 31 and an alanine at position 32 (when combining analysis of the SignalP and PrediSi software (16, 17)). Heterologous expression was conducted using pET28a as the expression vector and the full LOR_107 (LOR_107) or truncated LOR_107 (LOR_107d), the latter with the signal peptide deleted. The optimal temperature for overexpression induction and yield of soluble protein was found to be 16 °C (as compared with 37 and 25 °C). Protein expression was induced for 18 h, and the culture was fractionated by centrifugation to obtain the CM and cell pellet. The cell pellet was resuspended and disrupted, and then the cell lysate was collected. The two fractions, CM and cell lysate, were incubated with ulvan to determine ulvan lyase activity.

No ulvan lyase activity was detected in the culture supernatant of LOR_107d. Most of the enzyme activity was measured in the cell lysate, which accorded with the absence of signal peptide. Conversely, the LOR_107 supernatant exhibited strong ulvan lyase activity (Fig. 2, *A* and *B*). We used Western blotting analysis to detect the His-tagged proteins in fractions that showed ulvan lyase activity (Fig. 2*C*). The cell lysate of the LOR_107 contains two bands ~5 kDa apart, suggesting cleavage of the signal peptide. Furthermore, the apparent molecular mass of the secreted LOR_107 protein found in the CM is similar to the construct without the signal peptide (*i.e.* LOR_107d) found in the cell lysate, reinforcing our hypothesis that the enzyme is secreted.

Biochemical Characterization of a New Ulvan Lyase—^{1}H NMR of ulvan is presented in Fig. 3. The anomeric protons corresponding to iduronic acid (IdoUA-H1, 5.08 ppm), glucuronic acid (GlcUA, 4.58 ppm), and sulfated rhamnose (R3S, 4.90 and 4.82 ppm) were ascribed according to Lahaye and Robic (2). Following enzyme degradation, two intense signals appeared at 5.98 ppm (Δ-H1) and 5.46 ppm (Δ-H4) attributed to the unsaturated bond located at the non-reducing end of the end product of lyases. The peaks corresponding to the anomeric protons of the sulfated rhamnose residues linked to glucuronic acid resi-

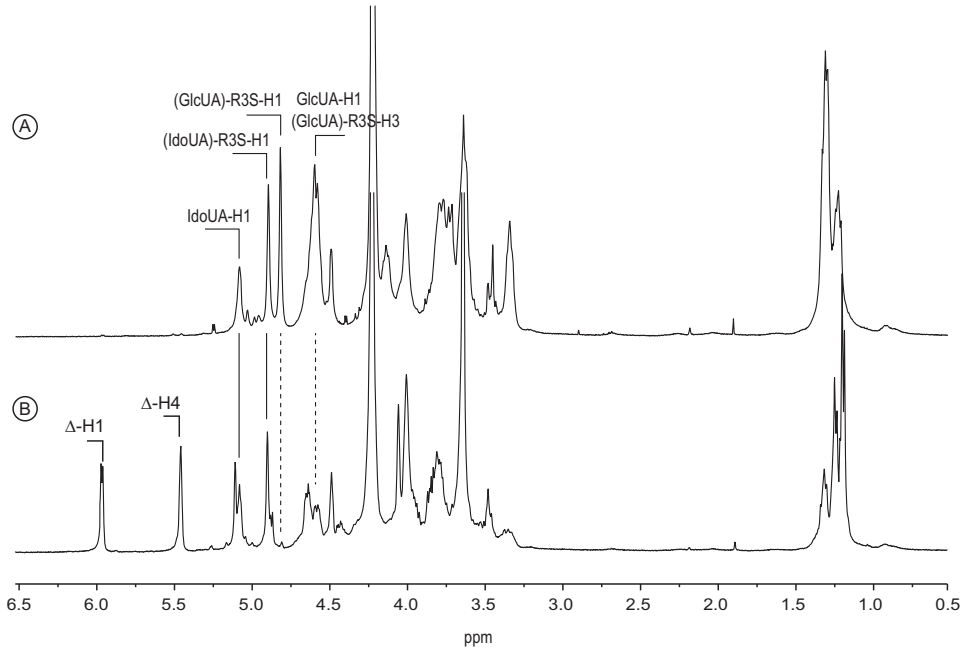

FIGURE 3. **^{1}H NMR spectra of ulvan degraded by LOR_107.** *A* and *B*, ulvan polysaccharide NMR spectra (*A*) as compared with end products spectra of ulvan incubated with purified LOR_107 (*B*). In spectra B, signals ascribed to the anomeric protons of the sulfated rhamnose residues linked to glucuronic acid residue (GlcUA-R3S-H1/H3) decreased strongly.

due (GlcUA-R3S-H1 4.82 and 4.9 ppm) decreased strongly. Similarly, the anomeric proton of the glucuronic acid residue (GlcUA-H1, 4.58 ppm) almost completely disappeared in contrast to the anomeric proton of the iduronic acid residue (IdoUA-H1), suggesting that the enzyme preferentially cleaves glucuronic acid residues.

Degradation kinetics of the ulvan lyase were followed at 235 nm to determine the optimal condition for activity. Initial velocity increased with temperature up to 40 °C; above this temperature, the enzyme began losing activity. The curve representing initial velocity as a function of pH showed a typical bell shape centered around pH 8. Variation of one pH unit led to a decrease of 8% in enzyme activity (data not shown).

Ulvan depolymerization catalyzed by LOR-107d ulvan lyase was monitored using gel permeation chromatography (Fig. 4). After the addition of enzyme, the molecular mass of ulvan, which eluted between 14 and 20 min of elution time, decreased rapidly, and intermediate oligo-ulvans appeared between 40 and 66 min of elution time (Fig. 4). After 24 h of incubation, the majority of the ulvan polysaccharides were converted into small-sized oligo-ulvans.

The ulvan lyase end products were purified and characterized by ^{1}H NMR according to Lahaye *et al.* (1). The most abundant end products eluting at 63 min were disaccharides attributed to the 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid linked to sulfated rhamnose (Δ-R3Sα/β, Fig. 5*A*). The second most abundant end product was a tetrasaccharide eluting at about 56 min, the structure of which was determined as Δ-R3S-IdoUA-R3Sα/β (Fig. 5*B*). A second minor tetrasaccharide was also purified, with the ^{1}H NMR characteristic of Δ-R3S-Xyl-R3Sα/β.

Characterization of Proteins Homologous to LOR_107 ulvan lyase—Based on identification of LOR_107, inspection of the genome of *Alteromonas* LOR revealed a second ulvan lyase,

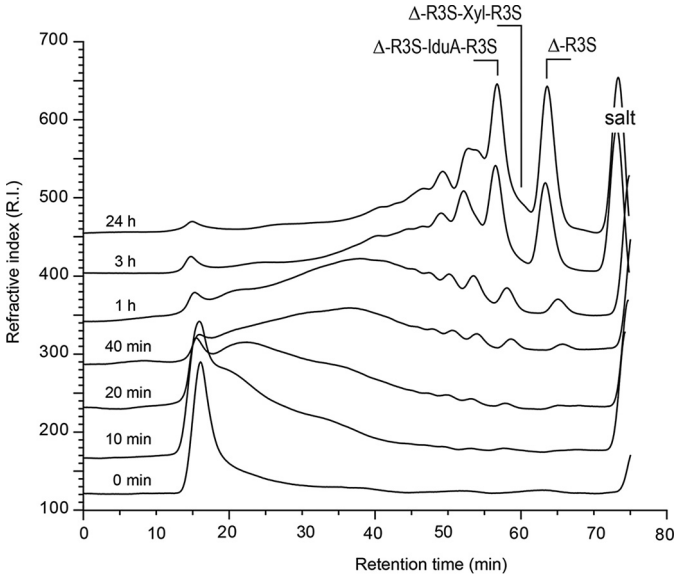

FIGURE 4. **Degradation kinetics of ulvan incubated with LOR_107 followed by gel permeation chromatography.** The high molecular weight ulvan polysaccharide is converted into oligo-ulvans rapidly. The two most abundant oligo-ulvans are disaccharides (Δ-R3S) and tetrasaccharides (Δ-R3S-Idu-R3S).

LOR_61. The molecular mass of the protein encoded by LOR_61 was estimated as 110.9 kDa, and the structure was predicted to comprise two modules when aligned with the short ulvan lyase (Fig. 6). The first module of 55.2 kDa was observed to share 68% sequence identity with LOR_107. The second module of 54.7 kDa at the C terminus was noted to resemble a conserved type II dockerin repeat domain (18) along with three repeating motifs of unclear function (p value < 0.0001) (19). The full protein was cloned and overexpressed successfully and, as expected, demonstrated ulvan lyase activity (Fig. 7).

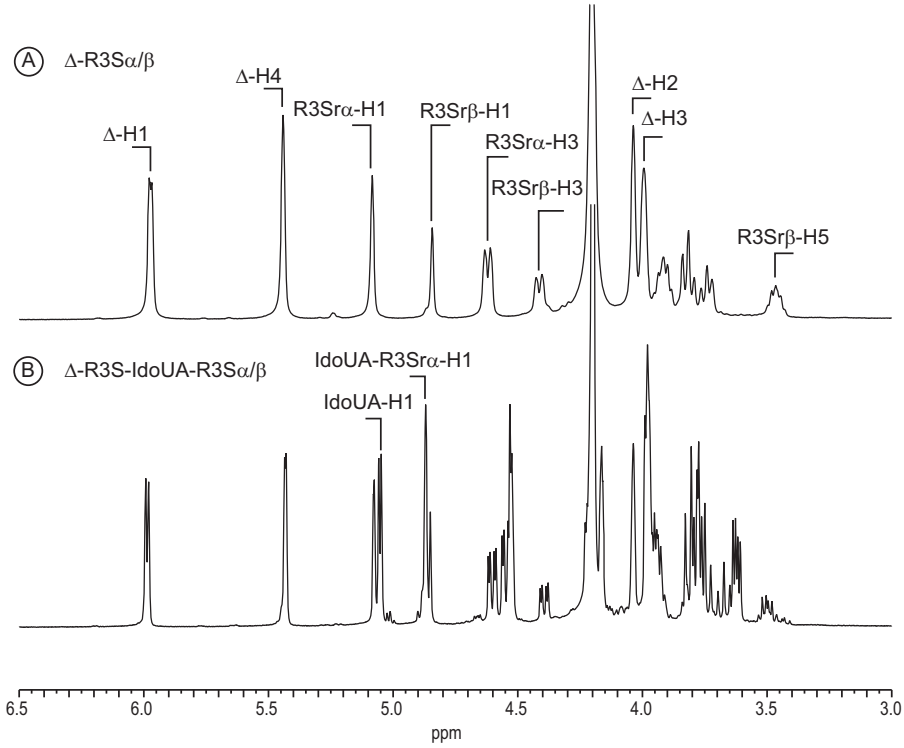

FIGURE 5. **^{1}H NMR of the purified most abundant LOR_107 end products.** *A*, spectra of the pure DP2 (Δ-R3Sα/β). *B*, spectra of the pure DP4 (Δ-R3S-IdoUA-R3Sα/β).

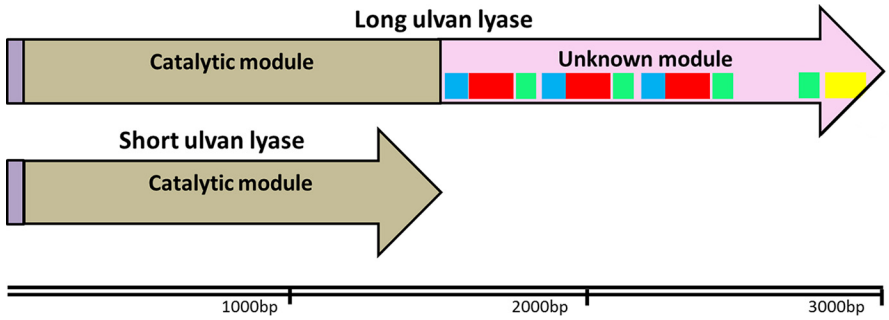

FIGURE 6. **Ulvan lyase sequence.** Long and short ulvan lyase sequence lengths are marked by the *arrows* (all peptide features are in scale). The sequence is divided into modules and domains: signal peptide (*purple*), catalytic module (*gray*), unknown module (*pink*), three motif domains (*blue*, *red*, and *green*), and type II dockerin domain (*yellow*).

Long and short ulvan lyases, homologous to LOR_61 and LOR_107, respectively, were found in *Alteromonas* LTR (LTR_2195, LTR_2241) and *Pseudoalteromonas* PLSV (PLSV_3925, PLSV_3875) (Table 3). *Alteromonas* LTR ulvan lyase sequences (LTR_2195, LTR_2241) are 100% identical to *Alteromonas* LOR ulvan lyases (LOR_61, LOR_107, respectively). The long protein similarly comprised an ulvan lyase catalytic module and a conserved type II dockerin. The proteins were overexpressed, and ulvanolytic activity was confirmed using gel permeation chromatography (Fig. 7). The end products were similar to those generated by LOR_107, suggesting common modalities of ulvan degradation.

Due to the low solubility of the purified overexpressed long ulvan lyases, biochemical characterization was performed on short ulvan lyases. The optimal temperature and pH for the three ulvan lyases were comparable, with only small variations observed. For example, temperature and pH optimum for PLSV_3875 was found to be 50 °C at pH 8, which differed only slightly from that observed for LOR_107. Notably, PLSV_3875 exhibited higher stability at elevated temperatures as compared with LOR_107, remaining 100% active after 24 h at 30 °C, whereas LOR_107 lost 90% of its activity in similar conditions.

The new ulvan lyase catalytic module sequence (Glu^{22}–Leu^{495} in LOR_61) was utilized to screen for other homologous sequences in the database. Similarity searches were performed using BLASTp (20) against all non-redundant (nr) protein databases. Sequence-based similarity results showed 128 sequences with significant identity (E-value $< 1 \times 10^{-4}$). Homologous ulvan lyases were found in several bacterial strains, with the highest sequence similarities (up to 88% identity found in *Pseudoalteromonas* sp. PLSV, E-value = 0.0) and in certain marine strains. The majority of the marine strains belong to Gammaproteobacteria, although surprisingly Flavobacteriaceae were found to possess putative proteins with 57% identity (E-value = 0.0). A few strains have multiple homologous proteins in their

genome. Specifically, *Catenovulum agarivorans* DS-2 is highly exceptional in that six homologous proteins were identified in the genome: four proteins with sequence identity greater than 62% and two others with greater than 44%.

Discussion

Putative protein-coding genes located in a gene cluster likely involved in carbohydrate metabolism were cloned and overexpressed, and the resulting proteins were screened for ulvanolytic activity. This targeted screening strategy enabled identification of a new ulvan lyase, LOR_107, with a protein sequence unrelated to the previously characterized *N. ulvanivorans* ulvan lyase (9). ^{1}H NMR analysis of the degradation end products, composed essentially of Δ-R3S and Δ-R3S-IdoUA-R3S, indicated that LOR_107 ulvan lyase cleaves specifically the

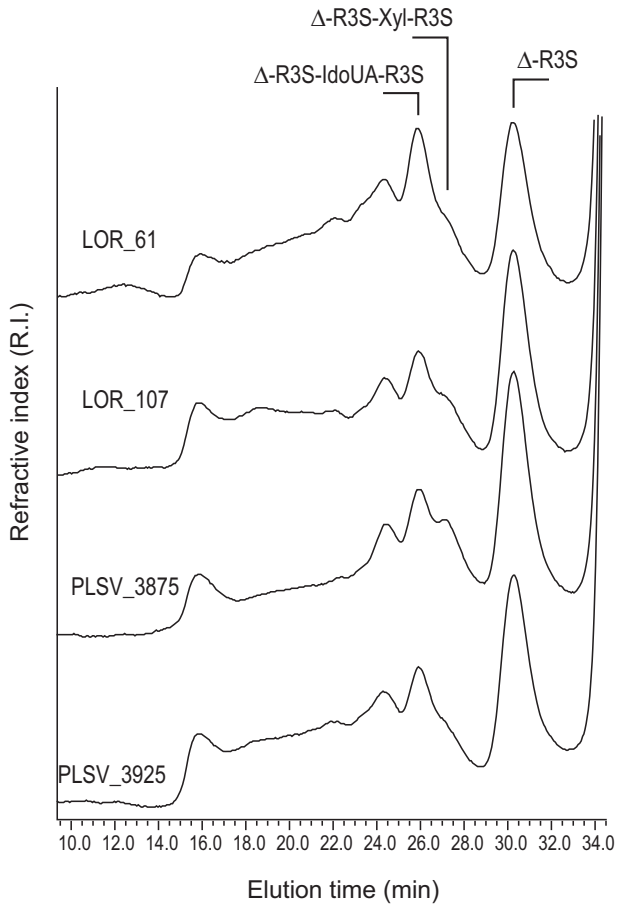

FIGURE 7. **Biochemical characterization of ulvan degradation by purified heterologous overexpressed ulvan lyases.** Gel permeation chromatography shows a comparison of the end products obtained after incubating ulvan with LOR_61, LOR_107, PLSV_3875, or PLSV_3925 ulvan lyases.

β(1→4) glycoside bond between GlcUA and R3S residues according to a β-elimination mechanism. This substrate specificity is a unique feature, as the previously identified ulvan lyase in *N. ulvanivorans* cleaves both GlcUA and IdoUA residues by abstraction of the proton on the C5 position in both configurations, *syn* or *anti*, with regard to the hydroxyl group on C4 (9).

Protein sequences homologous to LOR_107 were found in *Alteromonas* sp. LOR (LOR_61) and in two recently sequenced genomes: *Alteromonas* sp. LTR and *Pseudoalteromonas* sp. PLSV. Each of these proteins was shown to be an endo-ulvan lyase, producing end products similar to LOR_107. Taken together, these proteins represent the first members of a new family of biochemically characterized polysaccharide lyases. A bioinformatic search using the newly identified protein sequence indicated that many other putative proteins could be members of this novel lyase family. Further, based on the BLASTp results, it seems that this new family is much more abundant in bacterial genomes than the first ulvan lyase family identified in *N. ulvanivorans* (128 as compared with 20 sequences, respectively, E-value $< 1 \times 10^{-4}$). A key difference between the two ulvan lyase families is the distinctive recognition modalities allowing the LOR ulvan lyase to cleave specifically the glycoside bond between GlcUA and R3S, as opposed to the *N. ulvanivorans* ulvan lyase that cleaves both GlcUA and IdoUA attached to R3S. Furthermore, the ulvan lyase end products are degraded by β-glucuronyl hydrolase belonging to the GH105 family in *N. ulvanivorans* (11). In contrast, β-glucuronyl hydrolase belonging to the GH88 family appears proximal to the long ulvan lyase of *Alteromonas* LOR, LTR and *Pseudoalteromonas* PLSV. This proximity suggests that different enzymes are involved in the degradation of oligo-ulvans. Taken together, these observations raise the possibility that at least two different pathways of ulvan degradation have evolved independently to perform saccharification of ulvan.

In *Alteromonas* LOR, *Alteromonas* LTR, and *Pseudoalteromonas* PLSV, we found two homologous ulvan lyases: one "short" (59 kDa) and one "long" (110 kDa). The "short" ulvan lyase comprised the catalytic module preceded by a signal peptide, suggesting that it is exported outside the bacterial cell. Interestingly, we were able to show that *E. coli* can identify the *Alteromonas* signal peptide and cleave it while secreting a mature and active enzyme to the milieu. The "long" ulvan lyase in addition to the ulvan lyase catalytic module (*i.e.* the "short" segment) had a conserved type II dockerin repeat domain of about 6.7 kDa at the C-terminal of the protein. Dockerin domains are involved in anchorage of proteins at the cell surface by binding to cohesin (21). A module of about 48 kDa of unknown function was inserted between the catalytic and

TABLE 3

Molecular mass and GenBank accession numbers of the short and long ulvan lyases found in the genomes of *Alteromonas sp*. strains LOR and LTR, *Pseudoalteromonas sp*. PLSV and *N. ulvanivorans* PLR

Pair identity refers to the amino acid similarity between the catalytic modules of pairs of ulvan lyases (short and long).

Strains	Short ulvan lyase	Long ulvan lyase	Pair identity (in %)
Alteromonas sp. LOR	LOR_107 (59.6 kDa) *KU168251*	LOR_61 (110.9KDa) *WP_032096165.1*	68
Alteromonas sp. LTR	LTR_2241 (59.6 kDa) *KU168251*	LTR_2195 (110.9KDa) *WP_032096165.1*	68
Pseudoalteromonas sp. PLSV	PLSV_3875 (59.6 kDa) *KU168252*	PLSV_3925 (111.4KDa) *WP_033186955.1*	66
N. ulvanivorans PLR	PLR_48 (33.4 kDa) *WP_036579443.1*	PLR_42 (59KDa) *WP_036579437.1*	74

dockerin module. In this regard, it is notable that two ulvan lyases were also documented in *N. ulvanivorans*. The short version is composed of a catalytic module with signal peptide, whereas the long ulvan lyase also comprises a Por secretion system (recently renamed type IX secretion system) C-terminal sorting domain sequence. The C-terminal sorting domain mediates protein secretion by the type IX secretion system, a system that is unique to the Bacteroidetes phylum (22–25). *Flavobacterium johnsoniae*, which also belongs to the Bacteroidetes phylum, utilizes the type IX secretion system both for gliding motility and for extracellular chitinase secretion (26). As genes corresponding to the type IX secretion system apparatus were also found in the *N. ulvanivorans* genome, the C-terminal sorting domain sequence in the "long" *N. ulvanivorans* ulvan lyase likely serves to ensure secretion to the medium.

Full enzymatic conversion of ulvan to monosaccharides requires several more enzymes in addition to ulvan lyase and β-glucuronyl hydrolase, *e.g.* sulfatase, rhamnosidase, and xylosidase. Employment of a similar polysaccharide utilization locus screening strategy to that reported here may enable discovery of these enzymes. Further mining of the putative proteins may lead to the discovery of novel enzymatic activities and new GH families.

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